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Isolation of Different Bacteriophages Using the LamB Protein for Adsorption on *Escherichia coli* K-12

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Ten phages which use the LamB protein for adsorption have been isolated from sewage waters. Nine have a shape similar to lambda and require only the LamB protein for adsorption. One has a shape similar to T phages and can use either the LamB or the OmpC protein. Preliminary characterization by a number of criteria showed that at least nine of these phages were different and also differed from other known phages which use the LamB protein, such as lambda, 21, and K10.

The LamB protein forms an hydrophilic pore in the outer membrane of *Escherichia coli* K-12 (15). It allows the diffusion of low-molecular-weight molecules (<700) and is involved in the permeation of maltose and maltodextrins (for a review, see reference 7).

The LamB protein constitutes a specific receptor for phages lambda h^+ and its extended host range derivatives, lambda h, a one-step derivative, and lambda hh*, a two-step derivative with the most extended host range (9). It is used, in addition to lambda, by several other phages, i.e., K10, TP1, SS1 (1, 17, 19).

We have attempted to identify the regions in the LamB protein which are specifically involved in phage adsorption (3, 4). Such an approach is likely to improve our knowledge on what exactly constitutes a phage receptor site on the LamB protein and is likely to reveal regions of this protein located at the cell surface.

We are using this approach and report here the isolation and preliminary characterization of 10 phages which use the LamB protein for adsorption on *E. coli* K-12.

MATERIALS AND METHODS

Chemicals and media. Minimal medium M63B1, complete medium ML, and McConkey medium were previously described (5, 8). Phages were grown by the agar overlay technique (14), using tryptone agar (1% tryptone [Difco Laboratories], 0.025% NaCl, supplemented with 0.9% agar). Restriction enzymes *Eco*RI and *Hin*dIII were purchased from New England Biolabs, and isopropylthiogalactoside was from Sigma Chemical Co.

Bacterial and phage strains. Phage strains lambda b2vh, lambda b2vho, and lambda b2vhoh16 (9) were named for simplification lambda h^+, lambda h, and lambda hh*, respectively. Phage K10 was also used (6) for comparison with the new phages. Phage 21 EL is a clear plaque mutant of phage 21. It is a spontaneous mutant isolated by Elisabeth Brissaud after UV irradiation of a strain lysogenic for 21. The point mutations in *lamB* were in the genetic background of strain pop8 (Table 1), i.e., pop8.76 (*lamB5*), pop8.79 (*lamB*101), pop8.80 (*lamB*102), pop8.81 (*lamB*103), pop8.82 (*lamB*104), pop8.83 (*lamB*105), pop8.84 (*lamB*106), pop8.85 (*lamB*107), pop8.86 (*lamB*108), pop8.87 (*lamB*109), and pop8.90 (*lamB*112). The other bacterial strains used are listed in Table 1.

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Phage isolation. Phages were isolated from sewage waters of various origins. For each water sample, the same procedure was followed. A 10^{-2} dilution of the sample was plated on *E. coli* XA103. After 12 h of incubation at 37°C, 100 plaques of various sizes or shapes were chosen and tested; the plaques were picked with a tooth pick and tested on lawns of strains deficient in one or several outer membrane components as follows: P435 (LPS^-), CE1108 ($OmpF^-$ $OmpC^-$), CE1187 ($OmpF^-$ $OmpC^-$ $PhoE^-$), MH450 ($OmpF^-$), MH150 ($OmpC^-$), pop1737 ($LamB^-$), pop1737/h434^r/TuIa^r/TuII*^{r}($LamB^-$ $OmpC^-$ $OmpF^-$ $OmpA^-$), and on XA103.

The candidates unable to yield plaques only on strain pop1737 ($LamB^-$) and its multiresistant derivatives pop1737/h434^r/TuIa^r/TuII*^{r} were further tested. The corresponding plaques on XA103 were resuspended in 500 μl of 63B1 minimal medium. Chloroform (50 μl) was added, and the suspension was vortexed for a few seconds to inactivate any remaining bacteria. The phages were then purified on strain XA103. Four plaques from each purification were tested by spotting them on the various bacterial mutants previously used to recheck the phenotypes. Three to five purifications were usually necessary to obtain pure clones.

Eleven sewage samples were used to select the 10 phages. In three samples of the 100 plaques tested, no candidate could be selected. In six samples, one candidate was obtained. In two samples, two candidates could be isolated, AC43 and AC95 and AC50 and AC81.

Phage stock preparation. Phage stocks were prepared on strain XA103 by the plate lysate method. For each plate, 0.1 ml (≃10^6 PFU) of phage suspension was mixed with 0.1 ml of bacteria (≃10^8 bacteria per ml) and incubated for 20 min at 25°C. The mixture was then spread by the agar overlay technique (14) onto ML solid medium. After 9 to 14 h of incubation at 37°C, confluent lysis could be observed. For each phage stock, 30 plates were prepared in this way. Phages were then collected; the agar and the bacterial debris were removed by a short centrifugation (8,000 × *g* for 10 min at 4°C). The supernatant was submitted to a continuous cesium chloride gradient as described (13). Phages were then dialyzed against 10 mM Tris–1 mM $MgSO_4$–100 mM NaCl buffer and stored at 4°C.

Comparison of the frequencies of spontaneous phage-resistant mutants in strains XA103 and pop5234. For each phage, the following test was performed. Drops of phage and drops of lambda h^+ (about 50 μl of a suspension at 10^8 PFU/ml)

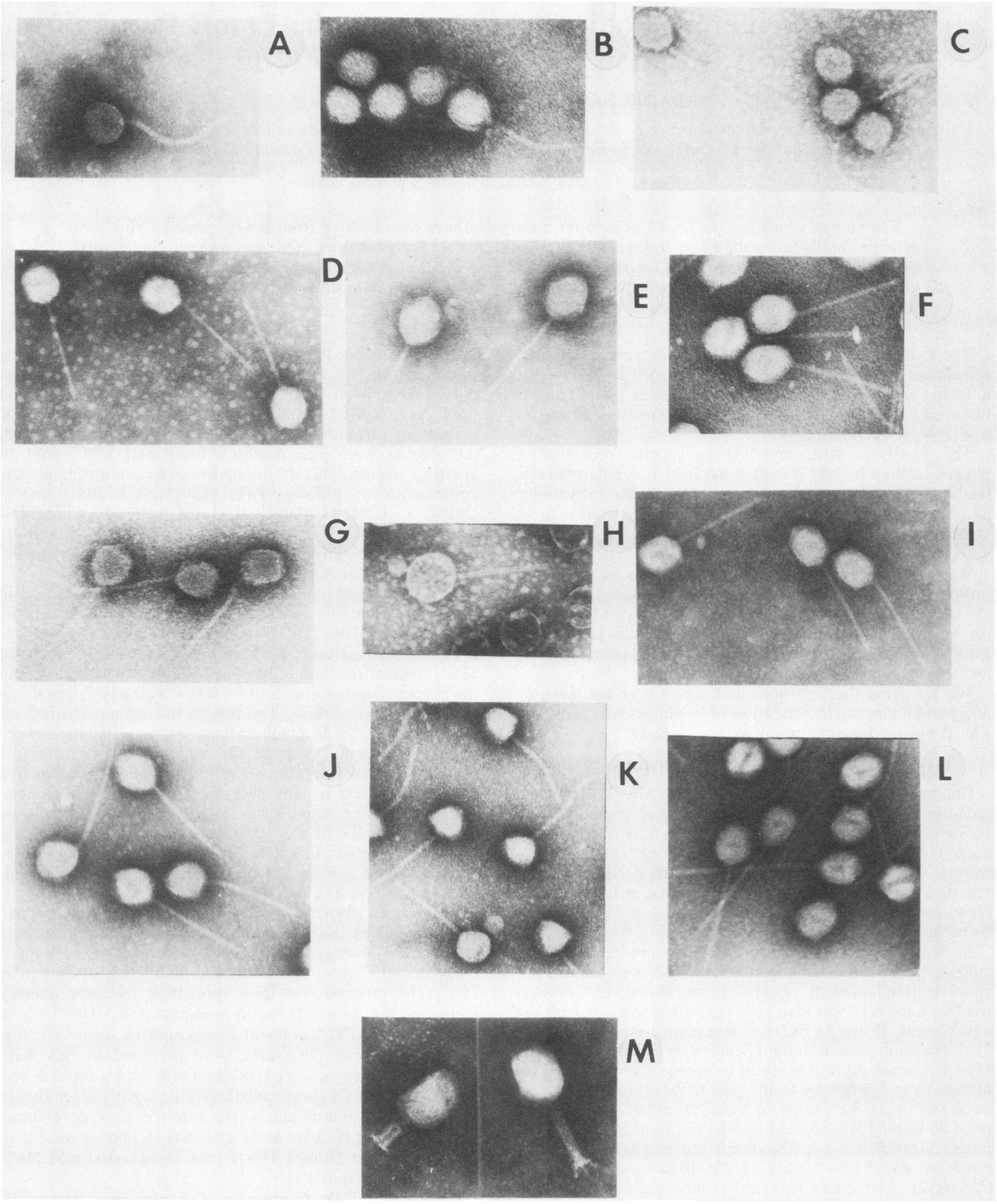

FIG. 1. Electron micrographs of the phages. (A) AC30, (B) AC7, (C) AC43, (D) AC95, (E) AC50, (F) 21EL, (G) AC6, (H) AC28, (I) AC57, (J) AC81, (K) lambda h^+, (L) K10, (M) AB48.

TABLE 1. List of strains

Strain	Characteristics useful for this study	Other characteristics	Source
pop8		F⁻ *thr leu thi argH metA str tonA*	Laboratory collection
XA103		F⁻ *argEamsupF*(Δ(*lac pro*) *nalA*ʳ *rif*ʳ *metB araD*	Laboratory collection
pop5234	F6 (*lac-lamB*) Mal⁺ carries an additional *lamB* gene in the *lac* region under *lac* promoter control	HfrC *thi rpoB*500	Laboratory collection
pop1737	*malB*Δ107 LamB⁻	Hfr G6 *his*	Laboratory collection
pop1737/H434/TuIa/TuII*	TuIaʳ h434ʳ TuII*ʳ	Derivative of pop1737	Laboratory collection
CE1108	*ompB*471 [OmpF⁻ OmpC⁻] *phoS* PhoE constitutive	*thr leu thy pyr*F *thy ilv his lacY argG tonA tsx rpsL cod dra vtr glp*	B. Lugtenberg
CE1187	*omp*B471 [OmpF⁻ OmpC⁻] *phoS* PhoE⁻ (TC45ʳ)	*pro* derivative of CE1108	B. Lugtenberg
MH450	*ompF*::Tn*5* OmpF (TuIaʳ)	F⁻ *araD*139Δ*lac rpsl relA thiA*	M. Hall
MH150	*ompC*::Tn*5* OmpC (TuIbʳ)	F⁻ *araD strΔ lac relA thiA FlbB*	M. Hall
P435	Hep⁻ LPS⁻, devoid of all lipopolysaccharide core sugars other than ketodeoxyoctonic acid	*rfa thr ara leu proA lacY galK nonxyl mtl argE thi str supE*	A. Pugsley
MH504	Spontaneous lambdaʳ Mal⁺ (AB48ʳ)	Derivative of MH450	This work

were spotted on lawns of strains XA103 and pop5234. After 12 h of incubation at 37°C, the number of resistant clones grown inside the two spots were compared. This number varied betwen 100 and 400 in strain XA103 for all the phages and for lambda h⁺. On strain pop5234 (diploid for gene *lamB*), no resistant clone could be observed in any of the spots.

Strain XA103 was plated on MacConkey maltose. Strain pop5234 on MacConkey maltose with 10^{-3} M isopropylthiogalactoside to get full induction of the *lamB* gene copy under lactose promoter control. Bacterial culture (0.1 ml) at 5×10^8 CFU/ml was spread on each plate.

Assay for phage inactivation. In vitro assays for phage inactivation were performed with the extract R prepared from strain pop725 (lambda sigma 3h434) as described previously (2).

Gel electrophoresis of phage major proteins. Electrophoresis of phage proteins was performed on a 15% acrylamide gel

TABLE 2. In vitro assay for phage inactivation, growth pattern on several *lamB* point mutants, and structural characteristics

Group and phage[a]	Inactivation by LamB extract[b]		Growth on *lamB*⁺	Growth on *lamB* point mutants[c]						Morphology		DNA profile[d]	Protein profile[d]
				Class I			Class I*, *lamB*110	Class II, *lamB*113	Class III, *lamB*102	Head length (nm)[e]	Tail length (nm)[e]		
	With ethanol	Without ethanol		*lamB*103	*lamB*101	*lamB*104							
A, AC30	+	−	+	R	R	I	S	R	R	79	185	≠	≠
B													
AC7	−	−	+	R	R	I	S	R	R	79	202	≠	≠
AC43	−	−	+	R	R	I	S	R	R	79	163	□	—[f]
AC95	−	−	+	R	R	I	S	R	R	79	167	□	□
AC50	−	−	+	R	R	I	S	R	R	92	202	○	≠
C, 21EL	+[g]	+[g]	+	I	R	R	I	R	R	88	176	≠	≠
D													
AC6	−	−	+	R	R	R	S	R	R	79	167	≠	≠
AC28	−	−	+	R	R	R	S	R	R	88	158	≠	≠
AC57	−	−	+	R	R	R	S	R	R	83	172	□	□
AC81	−	−	+	R	R	R	S	R	R	88	176	○	≠
h⁺	+	−	+	R	R	R	R	R	R	61	150	≠	≠
h	+	+	+	S	S	S	I	R	R				
hh*			+	S	S	S	S	S	R				
K10	−	−	+	S	R	R	S	R	R	83	194		≠

[a] The four groups (A, B, C, and D) of new phages are defined by their interactions with LamB (see the text). Of the mutational sites to lambda h⁺ resistance (3), only five were useful in distinguishing between the 10 phages which only used the LamB protein. *lamB*107, *lamB*108, *lamB*109, and *lamB*112 behaved like *lamB*101.

[b] +, Inactivation; −, no inactivation.

[c] Efficiencies of plating: S, ≈1; R, $<10^{-5}$; I, $>10^{-2}$ and $<10^{-1}$.

[d] ≠, Different from all other profiles; □, indistinguishable from □; ○, indistinguishable from ○.

[e] Usually 10 to 15 particles were measured.

[f] —, The protein profile is similar but may be different from those of AC95 and AC57.

[g] The rate of inactivation is much lower ($< 10^{-2}$) than that for lambda h (see the text).

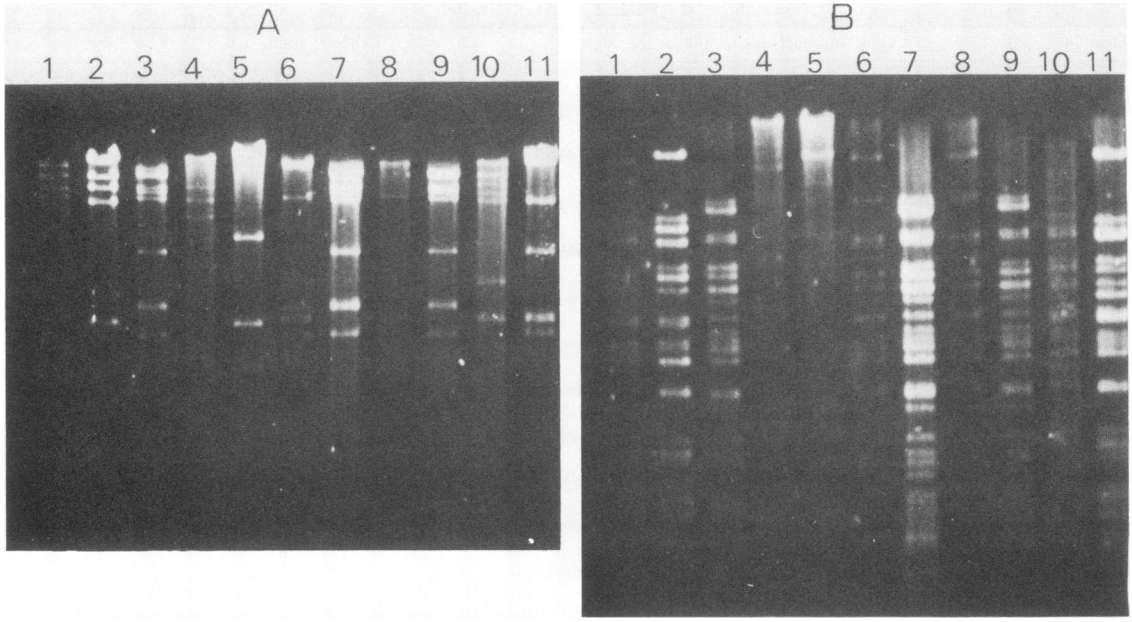

FIG. 2. Restriction patterns of phage DNAs. (A) the *Eco*RI profile, (B) the *Hin*dIII profile. Lanes (DNA of phages): 1, AC28; 2, AC7; 3, AC57; 4, lambda h^+; 5, 21EL; 6, AC81; 7, AC43; 8, AC50; 9, AC95; 10 AC6; 11, AC30.

prepared as described previously (11) and stained with Coomassie brilliant blue. A total protein amount of ≃5 μg was chosen for each phage sample. Samples were obtained from the phage stocks. The amount of proteins was calculated by the method of Lowry et al. (12).

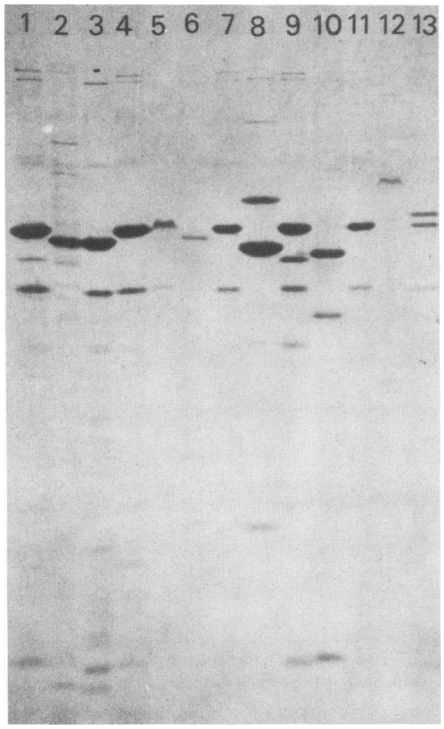

FIG. 3. The 15% acrylamide gel electrophoresis of phage proteins. Lanes (phages): 1, AC43; 2, AC28; 3, AC50; 4, 21EL; 5, AC7; 6, AC81; 7, AC95; 8, K10; 9, AC30; 10, lambda h^+; 11, AC57; 12, AB48; 13, AC6.

Phage DNA preparation and restriction analysis. DNA preparations were produced by using the small-scale plate lysate method described by Maniatis et al. (13); the restriction enzyme analysis electrophoreses were run on a 1% agarose gel.

Electron micrographs of the phages. Samples of purified preparations were deposited on grids, fixed with formaldehyde, and negatively stained with phosphotungstic acid as previously described (18). They were examined under a Siemens Elmiskop 101 electron microscope.

RESULTS AND DISCUSSION

Phage plaques were obtained from sewage samples on an *E. coli* strain with a wild-type *lamB* gene (XA103). Plaques were then tested on a series of *E. coli* strains deficient in one or several outer membrane components (see above for details). This screening allowed isolation of nine phages requiring the LamB protein for infection (AC6, AC7, AC28, AC30, AC43, AC50, AC57, AC81, and AC95) and one phage which is an optional user of either the LamB or the OmpC protein (AB48). AB48 is thus similar to SS1 and TP1, as was previously reported (1, 19). All 10 phages made clear plaques. Phage 21 (10), a temperate lambdoid phage known to require the LamB protein, was also examined in the rest of this study; a clear plaque mutant (21 EL) was isolated for this purpose (see above).

To determine whether membrane components other than the one looked at in the screening could be involved in the adsorption of these phages, the frequencies of bacterial mutants resistant to the phages were estimated in a strain diploid for gene *lamB* (pop5234; see above). Resistant mutants were not found, suggesting that no membrane component other than the LamB protein is required for infection by these phages.

Our next objective was to determine whether the 10 phages which used only the LamB protein for infection

could be distinguished on the basis of their interactions with this protein. Two approaches were used. First, the 10 phages were examined for their ability to grow on a set of well-characterized *lamB* point mutants (2). Ten different sites for tight missense mutations to phage lambda h^+ and for K10 resistance have been identified in gene *lamB* (3, 4). The growth pattern of the 10 phages on the 10 *lamB* missense mutants (Table 2) allowed us to distinguish three classes. Phages in class I (AC7, AC30, AC43, AC95, and AC50) made plaques on two of the missense mutants, *lamB*110 and *lamB*104. Phage 21EL, which constitutes class II, made plaques on *lamB*103 and *lamB*110. Phages in class III (AC6, AC28, AC57, and AC81) could only make plaque on *lamB*110. As expected, none of the phages made plaque on *lamB*102, an early nonsense mutant in *lamB*. In a second step, we examined the ability of an R extract (see above) of a $lamB^+$ strain to inactivate each of the 10 phages. R extracts of $lamB^+$ strains cannot inactivate in vitro phage lambda h^+. Addition of ethanol allows inactivation to proceed. Addition of ethanol is not required for inactivation of phage lambda h (16). Only two phages could be inactivated under the conditions used. Phage AC30 behaved like lambda h^+ (ethanol was required), and phage 21EL could be inactivated in the absence (or presence) of ethanol. However, for phage 21EL, concentrations of R extract had to be 100 to 1,000 times higher than those for lambda h to obtain the same rate of inactivation. Taking into account the previous results, the phages could be further classified into four groups: A, AC30; B, AC7, AC43, AC95, and AC50; C, 21EL; and D, AC6, AC28, AC57, and AC81.

We then examined whether the phages could be distinguished by their morphology or their DNA or protein contents. Electron microscopy observations showed that all 10 phages needing only the LamB protein for infection had a lambda phage shape and that phage AB48, the optional user of either the LamB or the OmpC proteins, had a T phage shape. According to criteria such as tail length, head length or morphology, and morphology of the tip of the tails, the 11 phages all appeared to be distinct, except perhaps phages AC95 and AC57 (Fig. 1), for which the difference in tail and head length may not be significant (Table 2). AC43 and AC95 are rather similar by most of these criteria, except that the morphologies of their heads seem different (Fig. 1).

Phage DNAs were submitted to restriction enzyme analysis. Each DNA preparation was digested separately with restriction endonucleases *Eco*RI and *Hin*dIII, and the restriction patterns were compared (Fig. 2). Seven different types of patterns were obtained, corresponding to phages AC30; AC7; AC43, AC95, and AC57; AC50 and AC81; 21EL; AC6; and AC28. Sodium dodecyl sulfate-acrylamide gel electrophoresis of total phage proteins (Fig. 3) showed that eight phages presented different patterns. The patterns for phages AC57 and AC95 were identical and similar to the one of AC43.

In conclusion, of the 10 phages examined which require only the lambda receptor for adsorption, 9 are clearly different by at least one of the criteria used (Fig. 1 and Table 2). Phages AC95 and AC43 could not be so unambiguously distinguished. As far as their interactions with the LamB protein are concerned, they constitute at least four different groups and are different from the phages which have been studied previously, such as lambda h^+, lambda h, lambda hh*, and K10. It is therefore hoped that they reveal new regions of the LamB protein involved in interactions with phages.

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